Phosphorylation and associated translocation of the 87-kDa protein, a major protein kinase C substrate, in isolated nerve terminals

(synaptosome/membrane association/myristoylation/phorbol ester/calcium)

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ABSTRACT A protein of 87 kilodaltons (87 kDa) was previously identified as a major specific substrate for protein kinase C in neuronal and other tissues. We have now studied the effect of protein kinase C-catalyzed phosphorylation of this protein on its association with membranes in isolated nerve terminals (synaptosomes) from rat cerebral cortex. Incubation of synaptosomal membranes under conditions associated with activation of protein kinase C led to the release of the phosphorylated 87-kDa protein into the incubation medium. In intact synaptosomes, activation of protein kinase C by phorbol esters or by depolarization-induced Ca^{2+} influx caused an increased phosphorylation of the 87-kDa protein and its translocation from membrane to cytosol. This translocation showed time courses, calcium dependency, and reversibility similar to those observed for the protein kinase C-induced phosphorylation of the protein. These results suggest that protein kinase C-catalyzed phosphorylation of the 87-kDa protein is responsible for its subcellular translocation into the cytosol of nerve terminals.

Protein kinase C is a widely distributed enzyme that is involved in a variety of neuronal functions (for reviews see refs. 1 and 2). The enzyme appears to be activated by Ca^{2+} - and diacylglycerol-dependent binding to membrane phospholipids (1, 3). It therefore seems likely that many physiological substrates for protein kinase C would be membrane-associated. An 87-kDa protein, originally observed as a substrate for protein kinase C in isolated nerve terminals (synaptosomes) (4), 3T3 cells (5), and broken cell preparations from rat brain (6, 7), represents a major, specific substrate for protein kinase C in a variety of cells and tissues (8, 9). When analyzed by ^{32}P -labeling, this phosphoprotein showed a predominantly cytosolic localization (6, 7, 10). When analyzed by immunoblotting, the 87-kDa protein in a synaptosomal fraction was predominantly membrane-associated (8). In murine macrophages, the membrane-associated form, but not the cytosolic form, of the 87-kDa protein appears to be myristoylated (11). The effect of phosphorylation of the 87-kDa protein on its subcellular localization was examined in the present study.

MATERIALS AND METHODS

Materials. ^{125}I -labeled protein A, $^{32}\text{P}_i$, and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ were from DuPont-New England Nuclear. Nitrocellulose membranes were from Schleicher & Schuell. Aprotinin was from Mobay Chemical; all other protease inhibitors were from Chemicon. Protein kinase C from rat brain was purified as described (12). An antibody was raised in rabbits against the 87-kDa protein purified from rat brain by the method described for bovine brain (13) and was found to recognize both

the phosphorylated and dephosphorylated forms of the protein (J.K.T.W., K. Albert, and P.G., unpublished results). All other chemicals were of reagent grade.

Preparation of Synaptosomal Membranes. A crude synaptosomal fraction (P_2) was prepared from rat cerebral cortex as described (14). This fraction was hypotonically lysed by resuspension in a glass/Teflon homogenizer with 10 up-and-down strokes in lysis buffer containing 10 mM Hepes (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, and the protease inhibitors aprotinin (50 kallikrein inhibitor units/ml) and leupeptin, antipain, and chymostatin (all at 10 $\mu\text{g}/\text{ml}$). Following centrifugation at $200,000 \times g$ for 20 min, the supernatant was discarded and the pellet was resuspended in lysis buffer by brief sonication and recentrifuged. The final synaptosomal particulate fraction was resuspended by brief sonication in lysis buffer to a final protein concentration of 2 mg/ml. Protein was measured by the method of Bradford (15), with bovine serum albumin as standard.

Analysis of the 87-kDa Protein in Synaptosomal Membrane Preparations. Aliquots (200 μg of total protein) of the membrane fraction were incubated (final volume, 0.2 ml) in 25 mM Hepes, pH 7.4/1 mM dithiothreitol/1 mM EGTA in the absence or presence of 10 mM MgCl_2 and 0.25 mM ATP, plus either 10 μM 8-bromo cyclic AMP, 1.5 mM CaCl_2 , or 1.5 mM CaCl_2 plus 10 nM purified protein kinase C. For ^{32}P -labeling of the 87-kDa protein, 5 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol; 1 Ci = 37 GBq) was added. After incubation at 30°C for 1 min, the reaction was terminated by the addition of a "stop" buffer containing (final concentration) 50 mM NaF, 5 mM NaPP_i, and 25 mM EDTA; and the incubation mixture was centrifuged at $200,000 \times g$ for 20 min. For immunoprecipitation of ^{32}P -labeled 87-kDa protein from the resultant supernatant and pellet fractions, NaDodSO₄ was added to both fractions to a final concentration of 1% (wt/vol), and the samples were heated in a boiling water bath for 2 min. The 87-kDa protein was immunoprecipitated with the antibody raised against purified rat brain 87-kDa protein and was subjected to NaDodSO₄/PAGE as described (8). With autoradiograms as guides, gel pieces containing the immunoprecipitated 87-kDa protein were cut out and ^{32}P incorporated into the protein was quantitated by liquid scintillation spectrometry.

For immunoblotting of the total 87-kDa protein in unlabeled samples, the supernatant and pellet fractions were subjected to NaDodSO₄/PAGE followed by electrophoretic transfer onto nitrocellulose (0.1- μm pore size) at 100 mA for 12–14 hr (8). The nitrocellulose membranes were probed with the anti-87-kDa protein antibody (1:500 dilution) and ^{125}I -labeled protein A, as described (8).

Preparation of Intact Synaptosomes. Synaptosomes were purified essentially as described (14), except that the four-step Percoll gradient was replaced by a three-step gradient consisting of 23%, 10%, and 3% Percoll. A synaptosome

fraction was collected from the 23%/10% interface, washed twice, and resuspended at a final protein concentration of 1 mg/ml in incubation buffer containing 140 mM NaCl, 5 mM KCl, 5 mM NaHCO₃, 1 mM MgCl₂, 10 mM glucose, 1.2 mM NaH₂PO₄, and 10 mM Hepes (pH 7.4).

Analysis of the 87-kDa Protein in ³²P-Labeled Synaptosomes. Labeling of purified synaptosomes with ³²P_i at 3 mCi/ml in phosphate-free incubation buffer was carried out as described (16), in the presence of either 0.1 mM CaCl₂ (Fig. 3) or 1 mM CaCl₂ (Fig. 4). Labeled synaptosomes (25 μg of protein) were incubated in the presence of 300 nM phorbol 12,13-dibutyrate (PDBu) or were depolarized by the addition of KCl to a final concentration of 40 mM (isotonicity being maintained by a corresponding decrease in the concentration of NaCl). Incubations were terminated by the addition of 10 volumes of chilled hypotonic buffer containing 10 mM Hepes (pH 7.4), 10 mM NaPP_i, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, and leupeptin, antipain, chymostatin, and pepstatin (all at 10 μg/ml). Samples were then centrifuged for 20 min at 200,000 × *g*, and the 87-kDa protein was immunoprecipitated from the supernatant and pellet fractions as described above.

Analysis of the 87-kDa Protein in Unlabeled Synaptosomes. Unlabeled synaptosomes (25 μg of protein) were subjected to PDBu treatment or K⁺ depolarization as described above for ³²P-labeled synaptosomes. After termination of the incubations with hypotonic buffer and centrifugation, the supernatants and pellets were subjected to NaDodSO₄/PAGE and immunoblotting for the 87-kDa protein was carried out as described above. A standard curve for immunoreactive 87-kDa protein, constructed with various amounts of synaptosomes, showed that the immunoreactivity measured in the experimental samples was within the linear range of the curve. Results are expressed as percent of total 87-kDa protein (supernatant plus pellet) in each sample. The amount of total 87-kDa protein did not vary between samples by more than 15%.

RESULTS

Phosphorylation-Induced Release of the 87-kDa Protein from Synaptosomal Membranes. When synaptosomal membranes were incubated with [γ-³²P]ATP in the presence of Ca²⁺ plus exogenous protein kinase C and then subjected to high-speed centrifugation, most of the ³²P-labeled 87-kDa protein was found in the supernatant (Fig. 1). A slightly smaller stimulation was seen in the presence of [γ-³²P]ATP and Ca²⁺ alone (data not shown). We conclude that this Ca²⁺-dependent effect was induced by endogenous protein kinase C, for several reasons: endogenous calmodulin was removed during isolation of the synaptosomal membranes; addition of Ca²⁺ plus calmodulin was not more effective than addition of Ca²⁺ alone; and gel electrophoretic analysis of phosphoproteins indicated that Ca²⁺/calmodulin-dependent protein kinases did not become autophosphorylated or activated under the conditions employed (data not shown). Addition of 8-bromo cyclic AMP did not induce phosphorylation of the 87-kDa protein (Fig. 1).

Incubation of synaptosomal membranes in the absence of Ca²⁺ and Mg²⁺/ATP, followed by high-speed centrifugation, showed that about 5% of the immunoreactive 87-kDa protein was present in the supernatant. Addition of Mg²⁺/ATP plus Ca²⁺ (but not of either Mg²⁺/ATP or Ca²⁺ alone) increased the amount of immunoreactive 87-kDa protein in the supernatant by 2.5-fold over control levels (Fig. 2). Addition of Mg²⁺/ATP plus Ca²⁺ plus exogenous protein kinase C increased the amount of 87-kDa protein in the supernatant 3-fold over control levels (Fig. 2). Addition of 8-bromo cyclic AMP did not induce the release of the 87-kDa protein (data not shown).

FIG. 1. Release of ³²P-labeled 87-kDa protein from synaptosomal membranes. Synaptosomal membranes (200 μg of protein) were incubated with [γ-³²P]ATP and 1 mM EGTA in the absence (control, CON) or presence of 10 μM 8-bromo cyclic AMP (8BrAMP) or of 1.5 mM Ca²⁺ plus 10 nM purified protein kinase C (Ca²⁺/PKC) for 1 min at 30°C. The reaction was terminated by the addition of "stop" buffer and centrifugation. ³²P-labeled 87-kDa protein was immunoprecipitated from the supernatant (A) and pellet (B) fractions. Positions and sizes (kDa) of standard proteins are indicated at left. The 87-kDa protein migrates at approximately 80 kDa in our present gel electrophoresis system. The experiment was repeated twice with similar results.

Phosphorylation and Translocation of the 87-kDa Protein in Intact Synaptosomes. The subcellular distribution of the 87-kDa protein was examined in isolated nerve terminals. Synaptosomes were labeled with ³²P_i and then depolarized with 40 mM KCl or treated with 300 nM PDBu. These treatments caused a 2-fold (KCl) to 3.5-fold (PDBu) increase in the phosphorylation of the 87-kDa protein, and most of the increase in ³²P-labeled 87-kDa protein was found in the soluble fraction (Fig. 3A).

Under control conditions, approximately 70% of the immunoreactive 87-kDa protein was located in the particulate fraction and approximately 30% was present in the soluble fraction of synaptosomes (Fig. 3D). Depolarization-induced Ca²⁺ influx or treatment with PDBu changed this distribution: approximately 36% and 50% of the immunoreactive 87-kDa protein was found in the soluble fraction following K⁺ depolarization or PDBu treatment, respectively (Fig. 3D). The amount of the 87-kDa protein found in the particulate fraction decreased concomitantly, with the total amount of the protein in the samples remaining unchanged. The effect of K⁺ depolarization was smaller than that of PDBu but was reproducible from experiment to experiment. Incubation of synaptosomes with the inactive (4α) isomer of PDBu did not change either the phosphorylation or the subcellular distribution of the 87-kDa protein when compared to control (data not shown).

Depolarization-induced phosphorylation and translocation of the 87-kDa protein in intact synaptosomes were dependent on the presence of Ca²⁺ in the extracellular medium (Fig. 3B and E). Furthermore, both the phosphorylation and translocation induced by K⁺ depolarization were reversed either by removal of extracellular Ca²⁺ (Fig. 3C and F) or by

FIG. 2. Release of immunoreactive 87-kDa protein from synaptosomal membranes. Synaptosomal membranes (200 μ g of protein) were incubated with 1 mM EGTA in the absence (control, CON) or presence of either 1.5 mM Ca^{2+} (Ca^{2+}), 10 mM Mg^{2+} /0.25 mM ATP ($\text{Mg}^{2+}/\text{ATP}$), 1.5 mM Ca^{2+} plus 10 mM Mg^{2+} /0.25 mM ATP ($\text{Ca}^{2+}/\text{Mg}^{2+}/\text{ATP}$), or 1.5 mM Ca^{2+} plus 10 mM Mg^{2+} /0.25 mM ATP plus 10 nM purified protein kinase C ($\text{Ca}^{2+}/\text{Mg}^{2+}/\text{ATP}/\text{PKC}$) for 1 min at 30°C. The reaction was terminated by the addition of "stop" buffer and centrifugation. The 87-kDa protein in the supernatant and pellet fractions was assayed by immunoblotting. Since the pellet fractions contained about 95% of the total 87-kDa protein and did not show significant changes under the various incubation conditions, results for only the supernatant fractions are shown. The experiment was repeated twice with similar results.

repolarization of synaptosomes through reduction of KCl concentration (data not shown).

Changes in phosphorylation and translocation of the 87-kDa protein after PDBu treatment showed similar time courses. Increases in both ^{32}P -labeled and immunoreactive 87-kDa protein in the supernatant were detected at the earliest time analyzed (15 sec), and the maximal increase was seen after 1–2 min of incubation of the synaptosomes with PDBu (Fig. 4). The decrease in the immunoreactive 87-kDa protein in the particulate fraction was also maximal within 1–2 min (Fig. 4).

DISCUSSION

The results of the present study indicate that the 87-kDa protein, a major substrate for protein kinase C, is released from the membrane when it is phosphorylated by protein kinase C in synaptosomal membranes or intact nerve terminals from rat cerebral cortex. Immunoblotting showed that a fraction of the 87-kDa protein was released from synaptosomal membranes when these membranes were incubated with ATP and endogenous or exogenous protein kinase C *in vitro*. Moreover, the use of [γ - ^{32}P]ATP showed that almost the entire pool of the phosphorylated 87-kDa protein was quantitatively released from the membranes.

In intact synaptosomes, a similar membrane-to-cytosol translocation of the phosphorylated 87-kDa protein was seen after PDBu treatment or K^{+} depolarization, both of which activate endogenous protein kinase C in the nerve terminals (4, 16). In intact synaptosomes, greater than 80% of the ^{32}P -labeled 87-kDa protein was found in the cytosolic fraction after phorbol ester treatment. By immunoblotting, approximately 20% of the total synaptosomal 87-kDa protein was

FIG. 3. Phosphorylation and translocation of 87-kDa protein induced by K^{+} depolarization or PDBu in intact synaptosomes. (Upper) Purified synaptosomes, labeled with $^{32}\text{P}_i$ as described in *Materials and Methods*, were centrifuged and resuspended in Ca^{2+} -free incubation buffer and preincubated at 37°C for 10 min under the following conditions: A, 1 mM CaCl_2 ; B, 0.1 mM EGTA added for the last minute of the preincubation; and C, 1 mM CaCl_2 . The synaptosomes were then incubated for 5 min with control buffer (CON), control buffer followed by 40 mM KCl for the last 15 sec (K^{+}), or 300 nM PDBu. For A and B, incubations were terminated by the addition of 10 volumes of hypotonic buffer and centrifugation. For C, EGTA was added to a final concentration of 2 mM at the end of the incubation period; the samples were then incubated another 10 min before the addition of hypotonic buffer and centrifugation. The 87-kDa protein was immunoprecipitated from the supernatant (shaded bars) and pellet (open bars) fractions. (Lower) For D, E, and F, purified unlabeled synaptosomes were treated as described for A, B, and C, respectively. The supernatant (shaded bars) and pellet (open bars) fractions were assayed for the 87-kDa protein by immunoblotting. The experiment shown is representative of three separate experiments, with triplicate determinations for each condition and SEM of less than 20%.

translocated from the membrane to the cytosolic fraction following phorbol ester treatment. The greater fold-increase in ^{32}P -labeled 87-kDa protein than in immunoreactive 87-kDa protein in the cytosolic fraction can be explained by the presence of unlabeled, and perhaps already phosphorylated, cytosolic 87-kDa protein in isolated synaptosomes prior to treatment with phorbol ester or K^{+} depolarization.

There was a good qualitative correlation between the state of phosphorylation and the translocation of the 87-kDa protein both in synaptosomal membranes and in intact synaptosomes. Translocation of the 87-kDa protein closely followed phosphorylation of the protein in its time course. Differences in efficacy and Ca^{2+} sensitivity between the phorbol ester-induced and the depolarization-induced phosphorylation were reflected in parallel differences in the translocation of the 87-kDa protein. Moreover, when depolarization-induced phosphorylation was reversed by removal of extracellular Ca^{2+} , there was a concomitant reassociation of the 87-kDa protein with the membrane.

These results are consistent with the interpretation that protein kinase C-catalyzed phosphorylation of the 87-kDa

FIG. 4. Time course of PDBu-induced phosphorylation and translocation of the 87-kDa protein in intact synaptosomes. (A) Purified synaptosomes were labeled with $^{32}\text{P}_i$ and incubated with 300 nM PDBu for the indicated times. The incubations were terminated by the addition of hypotonic buffer and centrifugation. The 87-kDa protein was then immunoprecipitated from the supernatant (●) and pellet (○) fractions. (B) Purified unlabeled synaptosomes were treated as described above for ^{32}P -labeled synaptosomes. The 87-kDa protein in the supernatant (●) and pellet (○) fractions was assayed by immunoblotting. The experiment shown is representative of three separate experiments, with triplicate determinations for each point and SEM of less than 20%.

protein in isolated nerve terminals probably takes place in association with membranes and that the phosphorylated protein becomes translocated from membranes to a soluble compartment *in situ*. Indeed, the correlation between phosphorylation and translocation of the 87-kDa protein strongly suggests that translocation may be causally related to the

degree of phosphorylation of the 87-kDa protein itself (although effects mediated through phosphorylation of other proteins cannot be ruled out). Previous studies have shown that, in murine macrophages, the myristoylated 87-kDa protein is predominantly membrane-associated whereas the 87-kDa protein phosphorylated as a result of activation of protein kinase C is predominantly cytosolic (11). Thus, myristoylation appears to be a major determinant of the membrane association of the 87-kDa protein. The present results from synaptosomal membranes and intact synaptosomes indicate that protein kinase C-catalyzed phosphorylation also regulates translocation of the 87-kDa protein. The exact relationship between phosphorylation and myristoylation of the 87-kDa protein, and the role(s) these post-translational modifications play in the subcellular localization of the protein, represents an important area for further study. Given the ubiquitous presence of the 87-kDa protein (8, 9), it will also be important to ascertain whether protein kinase C-catalyzed phosphorylation of the 87-kDa protein leads to cytosolic translocation of the protein in all cells and tissues.

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